(23) Brain hemorrhage.

Antisense Oligodeoxynucleotide Inhibition of Tumor Necrosis Factor-α Expression Is Neuroprotective After Intracerebral Hemorrhage

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Background and Purpose—Tumor necrosis factor-α (TNF-α) expression is increased in brain after cerebral ischemia, although little is known about its abundance and role in intracerebral hemorrhage (ICH). A TNF-α–specific antisense oligodeoxynucleotide (ORF4-PE) was used to study the extent to which TNF-α expression influenced neurobehavioral outcomes and brain damage in a collagenase-induced ICH model in rat.

Methods—Male Sprague-Dawley rats were anesthetized, and ICH was induced by intrastriatal administration of heparin and collagenase. Immediately before or 3 hours after ICH induction, ORF4-PE was administered directly into the site of ICH. TNF-α mRNA and protein levels were measured by reverse transcriptase–polymerase chain reaction and immunoblot analyses. Cell death was measured by terminal deoxynucleotidyl transferase–mediated uridine 5′triphosphate-biotin nick end labeling (TUNEL). Neurobehavioral deficits were measured for 4 weeks after ICH.

Results—ICH induction (n=6) elevated TNF-α mRNA and protein levels ($P<0.01$) at 24 hours after the onset of injury compared with sham controls (n=6). Immunohistochemical labeling indicated that ICH was accompanied by elevated expression of TNF-α in neutrophils, macrophages, and microglia. Administration of ORF4-PE (2.0 nmol) directly into striatal parenchyma, 15 minutes before (n=4) or 3 hours after (n=6) ICH, decreased levels of TNF-α mRNA ($P<0.001$) and protein ($P<0.01$) in the brain tissue surrounding the hematoma compared with animals treated with saline alone (n=6). Mean±SEM striatal cell death (cells per high-powered field) was also reduced in animals receiving ORF4-PE (34.1±5.0) compared with the saline-treated ICH group (80.3±7.50) ($P<0.001$). ORF4-PE treatment improved neurobehavioral deficits observed at 24 hours ($P<0.001$) after induction of ICH (n=6) compared with the untreated ICH group (n=6). This improvement was maintained at 28 days after hemorrhage induction ($P<0.001$).

Conclusions—These results indicate a pathogenic role for TNF-α during ICH and demonstrate that reducing TNF-α expression using antisense oligodeoxynucleotides is neuroprotective. (***Stroke***. **2001;32:240-248.**)

Key Words: behavior ■ intracerebral hemorrhage ■ neuronal death ■ oligodeoxyribonucleotide, antisense ■ tumor necrosis factor

Acute diseases of the central nervous system such as hemorrhagic and ischemic stroke, head injury, acute demyelination, and viral infection are often accompanied by inflammation, which may contribute to brain injury.[1,2] A key mediator of the inflammatory response is the proinflammatory cytokine tumor necrosis factor-α (TNF-α), although other cytokines such as interleukin-6 (IL-6) are increased during inflammation.[2] Under normal conditions, the expression of TNF-α is tightly regulated by rapid mRNA turnover.[3] However, during brain inflammation, macrophages and resident microglia are activated and produce elevated levels of TNF-α. TNF-α may also be released by astrocytes[4–6] and to a lesser extent from neurons, which constitutively express

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TNF-α.[7] Since TNF-α is produced by a number of cells during inflammation, it has been proposed to be a principal mediator of neurotoxicity during inflammation.[1,8,9] In agreement with this hypothesis, in vitro actions of TNF-α induce oligodendrocyte damage,[10] facilitate cytotoxicity of lipopolysaccharide-treated astrocytes and mediate astrocyte proliferation,[11] and may cause neuronal injury or death.[12]

Several reports indicate that TNF-α exacerbates focal cerebral ischemia, as evidenced by treatment with antibodies to TNF-α after brain injury, which significantly reduced infarct size.[1,8,13–15] Whether TNF-α acts directly or indirectly to induce neurotoxicity during ischemia, however, remains

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uncertain. Conversely, TNF-α has also been shown to be neuroprotective during ischemic stroke. Bruce and coworkers[16] have demonstrated that damage to neurons caused by focal cerebral ischemia and epileptic seizures was exacerbated in TNF-α receptor knockout mice, indicating that TNF-α may serve a neuroprotective function. In addition, TNF-α pretreatment induces protective effects against focal cerebral ischemia in mice,[17,18] further suggesting a protective role for TNF-α during brain injury.

Less is known about the pathogenic role of TNF-α during intracerebral hemorrhage (ICH), which accounts for 10% of all human strokes and is associated with 30% to 50% mortality within the first month.[19] ICH causes intense neutrophil and macrophage infiltration into the brain. This is correlated with increased TNF-α expression and increased brain damage.[20] To determine the role of TNF-α during ICH, the collagenase/heparin-induced ICH model in rats was used because it is accompanied by a pronounced and well-characterized inflammatory response.[21,22] TNF-α expression was initially defined in this model and subsequently modulated with the use of a previously reported TNF-α–specific antisense oligodeoxynucleotide (ODN),[23] administered both before and after the induction of ICH.

Materials and Methods

ICH Model

Sixty-two male Sprague-Dawley rats weighing 240 to 280 g were assigned to the experimental groups described in the Table. All animals were handled and cared for in accordance to the guidelines of the Canadian Council on Animal Care. Rats were anesthetized with pentobarbital (50 mg/kg IP) and placed in a stereotaxic frame (David Kopf Instruments). A 30-gauge needle was inserted through a burr hole into the striatum (location 3.0 mm lateral to the midline, 0.2 mm posterior to bregma, 6 mm in depth below the skull). ICH was induced by administration of 0.7 μL containing 0.14 U of collagenase (type IV; Sigma) and 1.4 U of heparin (Sigma) over 5 minutes.[21] Sham-operated animals were infused with the same volume of saline.

ODN Preparation

The TNF-α–specific ODN used in this study was previously characterized in our laboratory.[23] Briefly, ORF4-PE is a 29-mer oligodeoxyribonucleotide that is phosphorothioated on 3 nucleotides on each end of the molecule. ORF4-PE targets the second exon of TNF-α immediately upstream of the 5′ splice site. Previous studies showed that mismatched oligonucleotides homologous to ORF4-PE did not inhibit TNF-α synthesis in vitro and that the 21-mer ODN, O-8433, that targets the HIV-1 tat gene did not inhibit TNF-α synthesis in vitro.[23] O-8433 was therefore used in the present study to assess nonspecific actions of ODNs. All ODNs were synthesized by standard phosphoramidite methods at 0.05- or 0.2-μmol scales and were high-performance liquid chromatography purified by the manufacturer (Life Technologies).

ODN Administration

For administration of ODNs, 15 minutes before ICH a microinfusion pump (Sage Instruments) delivered 2 μL of ORF4-PE (1.0 or 0.1 nmol/μL dissolved in saline) into the site of the induced hematoma over a 5-minute period through a 30-gauge needle. To test the therapeutic efficacy of ORF4-PE after ICH, ICH was induced as described above, and the animals were removed from the stereotaxic frame. Animals were repositioned in the stereotaxic frame 3 hours later, and 2 μL of ODN (1.0 or 0.1 nmol/μL) was delivered to the identical stereotaxic coordinates as described above. After all procedures, rats were allowed to recover with free access to food and water and were tested for neurobehavioral deficits.

Experimental Groups

Treatments	Rats, n	ODN (ng)	Analysis*
Controls			
Nonoperated	6	None	B, R, P
	4	None	B (28 d), R
Sham-operated	6	Saline	B, R, P
15 min before ICH			
	4	ORF4-PE (0.2)	B, R
	4	ORF4-PE (2.0)	B, R, P
	4	O-8433 (0.2)	B, R
	4	O-8433 (2.0)	B, R
3 h after ICH			
	4	ICH+saline	B, MR, R, P, T
	6	ICH+saline	B (28 d), R
	6	ORF4-PE (2.0)	B, MR, R, P, T
	4	O-8433 (2.0)	B, MR, R, P, T
	6	ORF4-PE (2.0)	B (28 d), R
	4	O-8433 (2.0)	B (28 d), R

*Animals were scored for neurobehavioral deficits (B) and hematoma size and white matter edema by MRI (MR) and were killed at 24 hours. Brain tissue was analyzed for TNF-α mRNA levels by RT-PCR (R) and for protein by immunoblot (P), and cell death was estimated by TUNEL (T). Where indicated, neurobehavioral deficit scores at 24 hours after ICH up to 28 days are reported. Animals were killed 28 days after ICH, and brain TNF-α mRNA was measured by RT-PCR.

Brain Dissections and Tissue Preparation

Rats were killed at 24 hours or 28 days after the induction of ICH and neurobehavioral or MR testing. Each animal was killed by decapitation approximately 5 minutes after pentobarbital overdose (100 mg/kg IP), and brains were collected and analyzed for TNF-α mRNA and protein levels. Brains were dissected in the coronal plane through the needle entry site (identifiable on the brain surface), which corresponded to the approximate midpoint of the hematoma (approximately 0.2 mm posterior to bregma). The anterior portion, which contained half of the hematoma, was immediately fixed in 4% paraformaldehyde for further pathological examination. At 3 mm posterior to the original cut, the brain was again cut in the coronal plane, and the ipsilateral side (which contains approximately half of the hematoma) was immediately frozen in liquid nitrogen. Ipsilateral sections were again bisected through the middle of the hematoma at a right angle to the brain midline. Ventral portions of the ipsilateral sections were used for mRNA or Western blot analysis where indicated (Table).

TNF-α Immunohistochemistry

To define TNF-α expression and localization after ICH, animals from a separate study were anesthetized at different time points after collagenase/heparin injection or 24 hours after sham injection with saline and killed by perfusion with 4% paraformaldehyde in 0.1 mol/L PBS, as previously described.[21] Fixed brains were cut coro-

nally through the needle entry site, and slices including the hematoma site were embedded in paraffin. Sections (6 μm) were dewaxed and rehydrated in 0.1 mol/L PBS. After blocking steps, they were incubated with rabbit polyclonal (serum) anti–TNF-α diluted 1/500 in 0.1 mol/L PBS with 0.1% BSA (antibody provided by Dr Dwight Nance, University of Manitoba) overnight at 4°C followed by biotinylated goat anti-rabbit IgG, then streptavidin-peroxidase, then diaminobenzidine. Samples included sham (n=2), 30 minutes (n=2), 1 hour (n=3), 2 hours (n=3), 4 hours (n=2), 12 hours (n=2), 1 day (n=3), 2 days (n=3), 3 days (n=3), 1 week (n=4), 2 weeks (n=4), and 3 weeks (n=4) after ICH. TNF-α immunoreactivity was assessed in a semiquantitative manner in tissues surrounding the hematoma site. Intensity of labeling (none, weak, strong) and quantity of cells labeled (rare, <10 per 775-μm-diameter field; moderate, 10 to 50; many, >50) were judged by an observer blinded to the timing of the hematoma. The identity of labeled cells was based on morphological features, determined by an experienced neuropathologist (M.R.D.B.). To confirm the identity of TNF-α expressing cells as microglia, double labeling was performed on tissues from the 24- and 48-hour ICH animals. Rehydrated sections were incubated overnight with biotinylated RCA-1 lectin (1/400 dilution; Sigma) overnight at 4°C followed by streptavidin-peroxidase and diaminobenzidine to define the microglia. This was followed immediately by incubation with anti–TNF-α (1/5 dilution) for 1 hour at room temperature followed by Cy3-conjugated anti-rabbit immunoglobulin.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was purified from half of the ventral ipsilateral section closest to midbrain (approximately 0.05 g wet weight) by established methods.[24] The remaining brain tissue was stored at −80°C for Western blot analysis. Pilot studies were performed comparing different polymerase chain reaction (PCR) cycle number and input RNA concentrations to ensure that linear amplification of template occurred. PCR amplification of 2 μL of cDNA product was performed with the use of either rat TNF-α primers (forward 5′ AGG CGC TCC CCA AAA AGA TG 3′ and reverse 5′ TGG ATG GCG GAG AGG AGG CTG A 3′ yielding a product of 480 bp), IL-6 (forward 5′ TGT CTC GAG CCC ACC AG 3′ and reverse 5′ GTA GAA ACG GAA CTC CAG AAG AC 3′ yielding a product 424 bp), or GAPDH primers (forward 5′ GCT GGG GCT CAC CTG AAG GG 3′ and reverse 5′ GGA TGA CCT TGC CCA CAG CC 3′ yielding a product of 384 bp). PCR conditions for TNF-α and IL-6 were 25 cycles at 95°C for denaturation (60 seconds), 53°C for annealing (60 seconds), and 72°C for extension (60 seconds). Products were separated by agarose gel (1.4%) electrophoresis, transferred to a nylon membrane under alkaline conditions with the use of 0.4 mol/L NaOH and 1 mol/L NaCl, and probed with a randomly labeled ^{32}P-dCTP human TNF-α, mouse IL-6 (gifts from Dr R. McKenna, University of Calgary), or murine GAPDH cDNA (a gift from Dr P. Nickerson, University of Manitoba). Densitometric analysis of reverse transcriptase (RT)–PCR products was performed with the public domain program NIH Image (version 1.60).

Western Blot Analysis

Ventral ipsilateral brain was homogenized in Lamelli buffer (1% final concentration of SDS), protein content was quantified, and 20 μg was separated in a 10% gel by SDS-PAGE. Gels were fixed, fixed or stained with Coomassie blue, and subsequently transferred to nitrocellulose membrane and probed with polyclonal anti–TNF-α (1:1000; Genzyme). After Western analysis, antigens were visualized by enhanced chemiluminescence (Boeringher Mannheim), and abundance was quantified by densitometric analysis as described above.

TUNEL

With the use of 10-μm sections of fixed brain from each group, terminal deoxynucleotidyl transferase–mediated uridine 5′triphosphate-biotin nick end labeling (TUNEL) was performed as previously reported.[25] Sections were digested with 2 mg proteinase K at room temperature for 15 minutes, washed in PBS, and incubated with 2% H_2O_2 to inactivate endogenous peroxidase. After another wash in PBS, sections were immersed in terminal deoxynucleotidyl transferase buffer containing terminal transferase (0.3 U/μL) and biotinylated dUTP (0.04 nmol/μL). The biotinylated DNA was detected after incubation with streptavidin-peroxidase (1:1000) and diaminobenzidine. Six serial sections were analyzed by an observer blinded to the slide identity, during which the number of TUNEL-positive nuclei was counted in 6 random fields in the striatum and proximal cortex at a magnification of ×400.

Neurobehavioral Evaluation

An observer blinded to the identity of the rats evaluated behavior, beginning at 24 hours after induction of ICH, with reevaluation on days 4, 7, 14, 21, and 28. The tests used were followed as previously described.[21] Briefly, they included the following: (1) spontaneous ipsilateral circling behavior, graded from 0 for no circling to 4 for continuous circling; (2) contralateral forelimb and hindlimb retraction capability, graded from 0 for no retraction to 4 for retraction of both limbs; and (3) ability to walk a 70-cm-long×2.4-cm-wide wood beam, graded from 0 for normal movement along the beam to 4 for no movement or for a rat that fell off the beam. The behavioral score was reported as a cumulative score of the 3 tests, with a maximum total score of 12.

Magnetic Resonance Imaging

MRI was performed as described in detail previously,[21] with the use of a Bruker Biospec MSL-X 7/21 spectrometer. Briefly, to assess hematoma size in sham-operated controls and animals treated with ORF4-PE or O-8433 ODN, a set of 11 contiguous T2-weighted spin echo images (echo time, 20, 40, and 60 ms; repetition time, 1500 ms; slice thickness, 1 mm; matrix size, 256×256; field of view, 3.5×3.5 cm^2) was acquired in the coronal plane 24 hours after ICH induction. The area of the hematoma within the ipsilateral hemisphere was measured in each animal from a single MR image taken through the center of the hematoma by tracing the outline of each region on the MR imager workstation. The area traced calculates the defined region directly in square millimeters.[26] Changes in white matter hyperintensity were quantified by calculating the ratio of the MR image intensity in the corpus callosum to the intensity in the lateral neocortex contralateral to the hematoma in a slice 4 mm posterior to bregma, using the image obtained with an echo time of 60 ms.[26]

Statistical Analysis

Behavioral scores and densitometric analyses of autoradiographic profiles from RT-PCR product or immunochemiluminescent protein levels were compared statistically by ANOVA followed by Tukey-Kramer multiple comparison tests. Statistical comparisons of MR intensity measurements and TUNEL-positive nuclei counts were performed by 2-tailed Student's *t* test. For all tests, statistical significance was considered at the level of $P<0.05$ (Instat2, Graphpad Software).

Results

TNF-α Abundance and Localization After ICH

To define the time course and localization of TNF-α expression after ICH, TNF-α immunoreactivity was analyzed in rat brain sections surrounding the hematoma 30 minutes to 21 days after induction of ICH (Figure 1). In this and subsequent experiments there was no unexpected mortality. In sham-operated animals, immunoreactivity was weakly detectable in a subpopulation of large cortical neuron dendrites, but TNF-α immunoreactivity was not present in the striatum, ependyma, or white matter (Figure 1A). Similar findings were observed 1 to 2 hours after ICH. At 4 hours after ICH, rare small, round cells with minimal cytoplasm, likely leukocytes, near the margin of the hematoma were immunoreactive with anti–

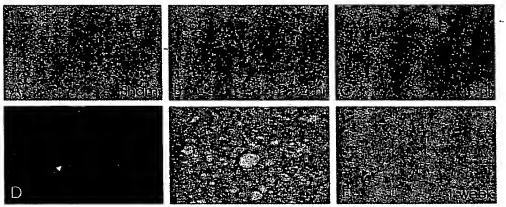

Figure 1. Figure 1. TNF-α immunoreactivity in striatum after ICH. Among sham animals, TNF-α–positive cells were not detected in the striatum (A). At 24 to 48 hours after ICH, some infiltrating neutrophils (B, arrowheads) as well as the ependymal cells near the hematoma (C) were immunoreactive. Double labeling showed colocalization of TNF-α immunoreactivity (D) and RCA-1 lectin detection (E) in many of the perivascular cells, which are considered microglia. At 7 days, intense microglial immunostaining was detectable around the hematoma (F, arrows). Magnification ×400.

TNF-antibody. Twelve hours after ICH, rare cells with flat dark nuclei and branched cytoplasmic processes characteristic of microglia were immunoreactive in the penumbra. Twelve hours after ICH, rare cells with branched cytoplasmic processes were immunoreactive in the striatum adjacent to the hematoma. At 24 hours a subpopulation (estimated 20%) of the neutrophils, which were identifiable by characteristic segmented nuclei, were immunoreactive. These infiltrated the surrounding striatal tissue and the margin of the hematoma (Figure 1B). The ipsilateral ependyma, immediately adjacent to the hematoma, was also strongly immunoreactive (Figure 1C). One and 2 days after ICH, many but not all microglia, identified by binding of RCA-1 lectin, in white matter and striatum near the hematoma were TNF-α immunopositive (Figure 1D and 1E). At 1 and 2 weeks, many macrophages in the hematoma, microglia in the penumbra (Figure 1F), and occasional ependymal cells still displayed TNF-α immunoreactivity. By 3 weeks after ICH, only rare hemosiderin-containing macrophages in the core of the lesion were TNF-α immunopositive (not shown).

Magnetic Resonance Imaging

To compare hematoma sizes, a set of 11 contiguous T2-weighted spin echo images were acquired 24 hours after ICH (Figure 2). Previous work showed that the hematoma size maximized by 4 hours, whereas white matter edema immediately posterior to the hematoma was maximal at 24 hours, with an approximate volume of 340 μL.[21] The hematoma size did not differ significantly in rats treated with saline (Figure 2A; 0.075±0.005 cm^2 [n=3]), ORF4-PE (2.0 nmol) (Figure 2B; 0.086±0.007 cm^2 [n=4]), or O-8433 (2.0 nmol) (Figure 2C; 0.099±0.002 cm^2 [n=4]) ($P<0.2$). ORF4-PE treatment of ICH-induced animals did not alter white matter edema at 24 hours after ICH ($P<0.4$). Specifically, the ratio of white matter hyperintensity between the corpus callosum and the contralateral neocortex was 1.97±0.02 in saline-treated animals (n=3), 1.92±0.02 in animals treated with ORF4-PE (n=4), and 1.92±0.02 in animals treated with O-8433 (n=4).

ORF4-PE Reduces TNF-α mRNA and Protein Production in ICH-Treated Animals

We previously showed that ORF4-PE specifically targets TNF-α mRNA in a sequence-specific manner in human and murine cell lines and does not affect the levels of expression of other cytokines, including IL-6.[23] To confirm that ORF4-PE targeted TNF-α mRNA in vivo, RT-PCR was performed on total RNA purified from ventral ipsilateral brain sections. Similar to the aforementioned immunocytochemical findings, TNF-α mRNA levels were significantly higher in the saline-treated ICH group than in the unoperated controls and sham (saline only) controls ($P<0.01$) (Figure 3). In agreement with cerebral ischemia models,[8,13,27–29] we found that elevated TNF-α mRNA levels were detected at 24 hours after ICH, as measured by RT-PCR (Figure 3). Animals receiving 2.0 nmol of ORF4-PE before ICH exhibited significantly lower levels of TNF-α mRNA compared with saline-treated animals ($P<0.001$), whereas animals receiving 0.2 nmol of ORF4-PE did not have significantly lower levels of TNF-α mRNA. Animals receiving O-8433 ODN exhibited TNF-α levels similar to those of ICH animals treated with saline (Figure 3). Animals receiving 2.0 nmol of ORF4-PE at 3 hours after ICH also had significantly lower levels of TNF-α mRNA compared with saline-treated animals (Figure 3). At 4 weeks after the ICH induction, there were no significant differences in TNF-α mRNA levels between groups (data not shown). IL-6 mRNA levels were significantly increased after ICH induction relative to sham animals (Figure 3C). However, ORF4-PE and O-8433 did not affect IL-6 levels in ICH animals (Figure 3D), suggesting that the actions of ORF4-PE were dose dependent and sequence specific.

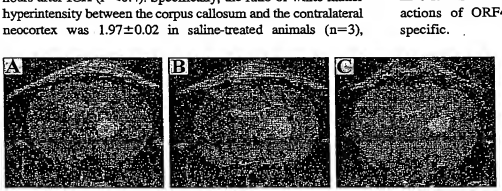

Figure 2. The size of the hematoma and the extent of white matter edema did not differ significantly between saline-treated (A), ORF4-PE–treated (B), and O-8433–treated (C) ICH animals, as represented by T2-weighted spin echo images at 24 hours after ICH.

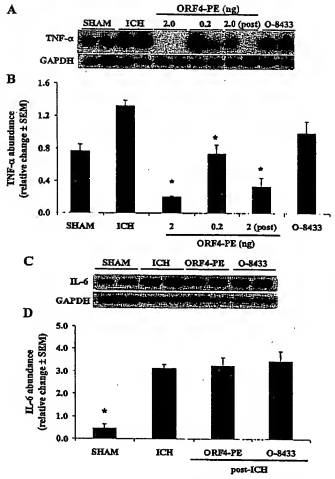

Figure 3. ORF4-PE administration significantly reduced ICH-induced TNF-α but not IL-6 mRNA detection at 24 hours after ICH. Animals treated with ORF4-PE 15 minutes before or 3 hours after ICH showed significantly lower levels of TNF-α mRNA in the striatum compared with ICH+saline-treated animals. A and C, Southern blot analysis of TNF-α or IL-6 and GAPDH RT-PCR products representing brain mRNA levels from the following groups: sham (animals without ICH); ICH (ICH+saline-treated animals); and ORF4-PE (ICH animals treated with 2.0 or 0.2 nmol of ORF4-PE before ICH or 2.0 nmol 3 hours after ICH); and O-8433 (ICH animals treated with 2.0 nmol of O-8433 at 3 hours after ICH). The figure represents 4 animals from each group. There was no significant difference in TNF-α mRNA levels between unoperated controls and sham-treated (saline alone) animals (RT-PCR not shown). B and D, Relative TNF-α or IL-6 mRNA abundance with reference to matched GAPDH levels. Although TNF-α levels were reduced in ORF4-PE–treated animals, IL-6 mRNA levels did not differ from saline-treated ICH animals. *$P<0.01$.

To confirm that the effects of ORF4-PE on TNF-α mRNA resulted in a reduction of TNF-α protein levels, TNF-α expression was compared among groups by Western blot in ventral portions of the ipsilateral brain, immediately posterior to the hematoma (Figure 4A). Consistent with the decreased levels of TNF-α mRNA, ORF4-PE treatment at 3 hours after ICH resulted in a significant reduction in TNF-α protein levels compared with TNF-α levels observed in saline-treated animals ($P<0.01$); in contrast, TNF-α levels were not affected by O-8433 (Figure 4B). Equal quantities of protein were present in each lane, as judged by Coomassie blue staining (data not shown).

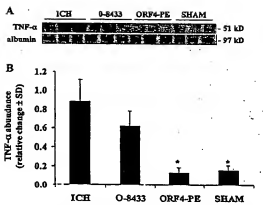

Figure 4. A, Immunoblot detection of TNF-α and albumin showed that ORF4-PE delivery (2.0 nmol) into the striatal parenchyma 3 hours after ICH significantly decreased TNF-α protein levels compared with sham controls, ICH+saline-treated (ICH), or O-8433–treated (2.0 nmol) animals. Sham animals received only saline without ICH. B, Relative TNF-α:albumin protein levels as determined by densitometric analysis. *$P<0.01$.

ICH-Induced Inflammation and Analysis of Cell Death

Histopathological examination of the tissues taken from animals killed 24 hours after the induction of ICH demonstrated blood in the striatum with foci of necrotic tissue in the center of the hematoma, edema in the surrounding tissue, and patchy inflammatory cell infiltrate composed largely of neutrophils. Neutrophil infiltration did not differ between experimental groups 24 hours after the onset of ICH (data not shown). To examine the extent of cell death after ICH in the aforementioned groups, TUNEL was performed on sections from sham animals (saline only) and ICH animals treated with saline, ORF4-PE (2.0 nmol), or O-8433 (2.0 nmol) (Figure 5). TUNEL-positive nuclei were rarely detected in the sham animals (Figure 5A). However, TUNEL-positive nuclei were easily detected in the striatum in the vicinity of the hematoma and, to a lesser extent, in the proximal cortex of saline-treated (Figure 5B) and O-8433–treated (Figure 5D) ICH animals, but the number of TUNEL-positive cells was reduced compared with animals treated with ORF4-PE (Figure 5C). Most of the labeled nuclei were small, localized to white matter bundles, and resembled glial cells. Larger TUNEL-positive cells were less frequently detected, although some, likely representing neurons, were observed in deep gray matter. Many of the small cells might be leukocytes; however, some nuclei were >15 μm in diameter and therefore could only be neurons. TUNEL-positive nuclei counts (Figure 5E) from sham animals or ICH animals treated with saline, ORF4-PE, or O-8433 showed that levels of TUNEL-positive nuclei were similar in the saline- and O-8433–treated groups but were significantly reduced ($P<0.001$) in both the striatum and proximal cortex in the ORF4-PE–treated group.

ORF4-PE Reduces ICH-Induced Neurobehavioral Deficits

To determine whether the ICH-induced pathological and molecular events that showed improvement after ORF4-PE treatment were paralleled by neurobehavioral recovery, repeated assessments of the animals were performed over a

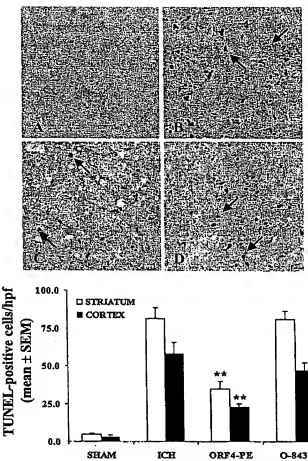

Figure 5. ORF4-PE treatment reduced TUNEL-positive cells in the region of the hematoma. Comparison of cell death in the striatum by TUNEL analysis in sham animals without ICH (A), ORF4-PE–treated (2.0 nmol) (C), and saline-treated (B) or O-8433–treated (2.0 nmol) (D) ICH animals. TUNEL-positive cells were rarely detected in the sham animals. In contrast, the number of TUNEL-positive cells (arrows) was higher in the saline-treated (B) and O-8433–treated (D) compared with ORF4-PE–treated animals (C). TUNEL-positive nuclei counts (E) revealed that ORF4-PE–treated animals displayed significant reductions in the striatum and proximal cortex compared with the saline- or O-8433–treated groups. Magnification ×200. $^{**}P<0.001$.

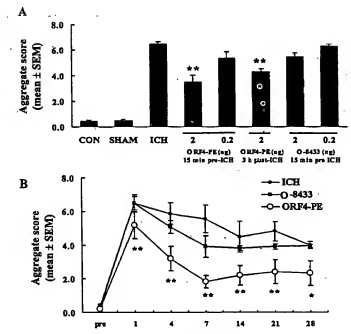

Figure 6. ORF4-PE treatment improves ICH-induced neurobehavioral deficits. A, ODN delivery 15 minutes before or 3 hours after ICH significantly improved the aggregate neurobehavioral deficits compared with sham controls or ICH+saline-treated animals. B, ORF4-PE treatment 3 hours after ICH induction significantly improved neurobehavioral deficits over a period of 28 days. Neurobehavioral deficits were scored on days 1, 4, 7, 14, 21, and 28 after ICH. Groups included the following: sham (animals treated with saline); ICH (ICH+saline); ORF4-PE (ORF4-PE [2.0 nmol] administered to animals after ICH as indicated); and O-8433 (O-8433 [2.0 nmol] administered to animals after ICH as indicated). Aggregate scores (mean±SEM) are from a minimum of 4 animals per group. See the Table for experimental conditions. $^{*}P<0.01$, $^{**}P<0.001$.

28-day period after ICH. ORF4-PE (2.0 nmol) delivered into the striatum 15 minutes before or 3 hours after ICH significantly improved animal neurobehavioral score at 24 hours after ICH ($P<0.008$) (Figure 6A) compared with the saline-treated ICH group. There was no statistical difference in neurobehavioral scores between groups receiving ORF4-PE (2.0 nmol) 15 minutes before or 3 hours after ICH (Figure 6A). In contrast, ORF4-PE (0.2 nmol) did not improve neurobehavioral deficits when delivered 15 minutes before or 3 hours after ICH (Figure 6A). Treatment with the nonspecific ODN, O-8433 (2.0 and 0.2 nmol), did not improve neurobehavioral outcomes (Figure 6A), suggesting that ORF4-PE actions were dose dependent and specific for TNF-α. ORF4-PE–treated animals (2.0 nmol) continued to show reduced neurobehavioral deficits at 1, 2, 3, and 4 weeks after ICH ($P<0.001$) (Figure 6B).

Discussion

Hemorrhagic stroke is accompanied by intense inflammation, including an initial infiltration by neutrophils followed by macrophage accumulation.[21] Both cell types express inflammatory molecules capable of damaging the central nervous system through a complex cascade of events.[8,28,30] Experimental and clinical cerebral hemorrhages induce a rapid production of proinflammatory cytokines, including TNF-α, in both brain parenchyma and cerebrospinal fluid.[29] Our studies showed that TNF-α gene expression is significantly elevated after ICH injury in specific cell types resembling neutrophils, ependymal cells, macrophages, and microglia. Intracerebral administration of a TNF-α–specific antisense ODN, ORF4-PE, both before and after ICH, resulted in several important outcomes, including the following: (1) dose-dependent and sequence-specific decreases in TNF-α, but not IL-6, levels that were consistent with the predicted actions of ORF4-PE; (2) decreased levels of cell death in the immediate area of the hematoma, as determined by TUNEL staining; and, (3) reduced neurobehavioral deficits at 24 hours that persisted up to 28 days after ICH. These observations suggest that the in vivo action of ORF4-PE, by limiting TNF-α expression, is neuroprotective during ICH.

The indirect neuropathological actions of TNF-α after ICH may be mediated by multiple mechanisms.[8] For example,

ICH-induced elevation in TNF-α may impair the ability of astrocytes to remove extracellular glutamate through dysregulation of calcium homeostasis, as shown for other neurological diseases, including HIV[31,32] or ischemia.[33] TNF-α may also act on microglia and macrophages to increase proinflammatory cytokine production via activation of nuclear factor-κB.[34,35] This would enhance reactive oxygen species, including nitric oxide.[36,37] In support of an indirect role for TNF-α–induced neuropathology, inhibition of free radical production during ICH by dimethylthiourea or α-phenyl-*N*-*tert*-butyl nitrone significantly improves neurobehavioral outcome,[26] suggesting that free radicals may play an important role in the development of brain injury after ICH. Clearly, identification of the cellular events mediated by TNF-α that potentiate neurotoxicity may elucidate new pathways that can be therapeutically targeted to ameliorate hemorrhagic brain injury.

Despite our findings that TNF-α has a pathogenic role during ICH, it is clear that basal levels of TNF-α are essential for normal growth and development, including neuronal and glial maintenance and survival.[16] Several researchers have shown that the role of TNF-α during ischemic stress is neuroprotective.[15–17] Hence, it appears that constitutive levels of TNF-α are required at all times in the brain parenchyma. In conditions during which central nervous system levels of TNF-α are abruptly elevated, such as ischemia,[8,28] hemorrhagic stroke,[20,29] or multiple sclerosis,[38] it may be desirable to mitigate the harmful effects of TNF-α, which may initiate a neurotoxic cascade.[28,30] In the present study ORF4-PE diminished TNF-α expression such that TNF-α levels were significantly reduced after ICH induction, yet TNF-α protein production was not completely eliminated in vivo (Figure 4). Indeed, this feature of ODN action is important because it enables efficient regulation of TNF-α without eliminating the constitutive levels that are required for cell survival. In TNF-α receptor knockout mice in which focal ischemia or epileptic seizures are induced, brain injury is worsened,[16] likely because of the lack of TNF-α receptors that would mediate the actions of basal levels of TNF-α, necessary for neuronal response(s) to pathophysiological stresses.

MRI results at 24 hours after the induction of ICH showed that hematoma size or white matter edema was not influenced by antisense treatment. Similarly, the extent of neutrophil infiltration was not altered, as judged by histological analysis (not shown). In contrast, cell death as estimated by semiquantitative TUNEL analysis showed fewer dead cells after treatment with ORF4-PE. These findings indicate that the magnitude of the initial cerebral injury was similar for all groups and, additionally, that the cellular events occurring during ICH are not always apparent by routine MRI. Our results also imply that neurobehavioral deficits after hemorrhagic stroke in rats may not be correlated with hemorrhage size or the extent of white matter swelling; rather, elevated proinflammatory molecule(s) production, including TNF-α, may be more predictive of neurobehavioral outcome. In addition, the specific region within the brain that is damaged is likely to be a more important determinant of functional deficits than the actual size of the lesion.[39] Other methods such as MR spectroscopy or diffusion-weighted imaging may help to further evaluate the nature and degree of brain damage after ICH.

In the present study immunocytochemistry showed that within the first 24 hours, the major source of TNF-α was derived from infiltrating neutrophils. However, at later time points, TNF-α was also expressed by pericytes and infiltrating leukocytes. Since we did not use a specific ODN carrier or conjugated ligand to deliver the antisense molecules to distinct cell types, the ODN was likely phagocytosed by multiple cell types, including microglia and infiltrating macrophages and neutrophils, which are known to engulf ODNs.[40] Since these cells are major producers of TNF-α during inflammatory events,[2] inhibition of TNF-α by antisense ODNs within these cells may have led to the observed significant reduction in TNF-α mRNA and protein levels. However, future experiments are necessary to confirm which cells within the hematoma are engulfing the antisense ODNs and the extent to which phagocytosis is dependent on the individual ODN.

Regardless of the cellular source of TNF-α production after ICH insult, the present study indicates that inhibition of TNF-α synthesis within the first 24 hours after ICH significantly improved neurobehavioral outcome. Furthermore, neurobehavioral improvement persisted for up to 28 days after ICH, which was accompanied by reduced cell death, perhaps through a mechanism involving apoptosis. Indeed, the present studies also suggest that although ORF4-PE acts immediately to selectively reduce TNF-α expression, the concentration of ODN at the site of hemorrhage was approximately 2 μmol/L, indicating that ORF4-PE is effective at concentrations comparable to those used in vitro.[23] The present study has demonstrated the possibility that the use of an ODN targeting TNF-α may offer an effective treatment for hemorrhagic stroke. However, it is important to establish efficient routes of administration other than direct intracerebral injection and to define the maximum time after hemorrhage at which treatment remains effective.

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Editorial Comment

The use of antisense oligonucleotides (AONs) for inhibition of gene expression provides a powerful method for investigating the function of specific proteins. AONs are also showing promise as a potentially new therapeutic modality. To date, these molecules have been applied in several different fields, including oncology, hematology, and cardiovascular and infectious diseases.[1] Because AONs do not pass the intact blood brain barrier (BBB), the only way to utilize these reagents in central nervous system (CNS) research is to administer them directly into the brain parenchyma or into the cerebral ventricles. Diverse targets have been explored with AONs for physiological and pathological functions in the CNS. These include neurotransmitter receptors, neuropeptides, trophic factors, transcription factors, cytokines, and other proteins.[2] Similar to other reports on the role of TNFα in cerebral injury (for review, see Barone and Feuerstein[3]), the authors demonstrate that an AON to TNFα confers neuroprotection in rats subjected to intracerebral hemorrhage (ICH) induced by disruption of the BBB integrity by collagenase. However, it is important to note that this approach harbors some pitfalls and limitations. First, side effects of AONs in the brain include potential nonspecific functional

neuronal alterations and detrimental effects on cellular morphology.[2] Recent advances in AON chemistry have significantly decreased these side effects.[4] Second, antisense therapeutic action against acute conditions such as ICH or stroke (where the therapeutic window is relatively short, 6 to 12 hours) may be best achieved with protein targets with relatively fast turnover time. Proteins that persist for longer periods ($T_{1/2}$ of days or weeks) may not be sufficiently inhibited during the narrow time window available for intervention.

Third, while it is commonly believed that the brain is somewhat "immune secluded" (ie, an organ of limited capacity to launch immune reactions), it has not yet been established whether a synthetic AON (a xenobiotic) delivered into the brain may ultimately stimulate immune competent cells to generate antibodies against RNA and/or DNA, triggering inflammation, with severe CNS consequences. Such possibilities, even though not yet fully explored, should be kept in mind. Fourth, specificity of action is a key issue, because interference with transcription and translation may result from the tendency of some AON chemistries to bind nonspecifically to proteins. In addition, hybridization of the AON to mRNA species other than the intended target, albeit suboptimally, might further confound the interpretation of the data. It is critical that large therapeutic indices be established in AON studies. The key demonstration, neuroprotection against ICH by an anti-TNFα modality, is a significant result even though AONs may not be a therapeutic option at this time. The clear reduction in functional deficits and salvage of cells and tissue 3 hours after the ICH event suggests potential benefits through interference with TNFα production. Recently, small organic molecules that inhibit tumor necrosis alpha converting enzyme (TACE) with great potency and selectivity have been synthesized and are being developed by pharmaceutical houses. Such small molecules may have a better chance of gaining access to the CNS and therefore may be delivered systemically or orally. It would be of interest to examine such pharmacological agents in ICH and other brain injury models in which TNFα may be a major mechanism leading to tissue damage and functional deficits.

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